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Chaperone protein GrpE and the GroEL/GroES complex promote the correct folding of tobacco mosaic virus coat protein for ribonucleocapsid assembly in vivo

D.-J. Hwang*, N. E. Tumer, and T. M. A. Wilson**

AgBiotech Center, Cook College, Rutgers University, New Brunswick, New Jersey, U.S.A.

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Summary. Several prokaryotic chaperone proteins were shown to promote the correct folding and in vivo assembly of tobacco mosaic virus coat protein (TMV CP) using a chimaeric RNA packaging system in control or chaperone-deficient mutant strains of *Escherichia coli*. Mutations in *groEL* or *dnaK* reduced the amount of both total and soluble TMV CP, and the yield of assembled TMV-like particles, several-fold. Thus both GroEL and DnaK have significant direct or indirect effects on the overall expression, stability, folding and assembly of TMV CP in vivo. In contrast, while cells carrying a mutation in *grpE* expressed TMV CP to a higher overall level than control *E. coli*, the amounts of both soluble CP and assembled TMV-like particles were below control levels, suggesting a negative effect of GrpE on overall CP accumulation, but positive role(s) in CP folding and assembly. Curiously, cells with mutations in *groES* and, to a lesser extent, *dnaJ* expressed total, soluble and assembled forms of TMV CP significantly above control values, suggesting some form of negative control by these chaperone proteins. To avoid pleiotropic effects or artefacts in chaperone-null mutants, selected chaperone proteins were also over-expressed in control *E. coli* cells. Overproduction of GroEL or GroES alone had little effect. However, co-overexpression of GroEL and GroES resulted in a two-fold increase in soluble TMV CP and a four-fold rise in assembled TMV-like (pseudovirus) particles in vivo. Moreover, TMV CP was shown to interact directly with GroEL in vivo. Together, these results suggest that GrpE and the GroEL/GroES chaperone complex promote the correct folding and assembly of TMV CP into ribonucleocapsids in vivo.

Present addresses: *Division of Molecular Genetics, National Institute of Agricultural Science and Technology (NIAST), Rural Development Administration (RDA), Suwon, Republic of Korea; **Scottish Crop Research Institute, Invergowrie, Dundee, U.K.

Introduction

Tobacco mosaic virus (TMV) is type member of the *Tobamovirus* genus of positive-strand RNA plant viruses. TMV has long been a model for the spontaneous self-assembly of complex, multimeric, biological structures in vitro [5, 6]. TMV assembly in vitro is initiated by a specific interaction between a prefabricated aggregate of 34 or more coat protein (CP) subunits and the origin-of-assembly sequence (OAS) located about 1 kb from the 3′-end of the genomic RNA in the common (U1) strain of TMV [41]. Nevertheless, little or nothing is known about the folding and assembly of TMV CP or other plant virus capsid proteins in vivo.

Previously, Haynes et al. [19] and Shire et al. [30] over-expressed U1 TMV CP in *Escherichia coli*. Following cell lysis and fractionation, this TMV CP could be polymerized in vitro, at low pH. Recently we showed that protein-only rods assembled in vitro from *E. coli*-expressed TMV CP differed structurally from those made with virus-derived CP and consisted of non-helical (i.e. planar) stacks, probably the so-called "disk" aggregates [20]. The propensity for *E. coli*-made TMV CP, which lacks the N-terminal acetyl group found in planta [30], to form planar bi-layer disks and stacked disks may be due to interlayer, intersubunit interactions between the free amino and carboxy termini (G. Stubbs, pers. comm.). We developed an in vivo assembly system for TMV-like particles in *E. coli* by co-expressing TMV CP and chimaeric ssRNAs containing the cognate OAS [20]. As *E. coli*-made TMV CP could encapsidate OAS-containing RNA molecules in vivo, but not in vitro, we proposed that some cellular factor(s) might enhance the folding and assembly processes of TMV CP in vivo.

The correct folding and assembly of many eukaryotic and prokaryotic cellular proteins, and some virus-coded proteins, require the assistance of molecular chaperones [17], which include heat shock proteins such as Hsp70 and Hsp60 [8, 13, 34, 40]. In *E. coli*, DnaK (an Hsp70-homologue) maintains proteins in an unfolded state, thereby preventing non-productive folding reactions. Later, ATP-dependent substrate release is controlled by the specific cofactors, DnaJ and GrpE which act co-operatively to stimulate the ATPase activity of DnaK [24]. *E. coli* GroEL (an Hsp60-homologue) is known to promote the correct folding of monomeric proteins such as rhodanase [27] and citrate synthase [4] and the assembly of oligomeric proteins such as ribulose bisphosphate carboxylase [14] and luciferase [9]. GroES acts as a cofactor to release substrate protein bound to GroEL [14, 26]. In contrast, several proteins can bind to GroEL but their folding is not improved and is independent of GroES; these include lactate dehydrogenase [3], dihydrofolate reductase [15, 36], and β-lactamase [23, 39]. Therefore, whether or not molecular chaperones assist folding and assembly depends on the identity of the substrate protein. It is also known that *E. coli* chaperones affect the folding and assembly of proteins encoded by bacteriophage λ, T4 and T5 [40].

Several plant viruses, such as citrus tristeza and beet yellows closteroviruses, have been shown to encode Hsp70 and Hsp90 homologues [1, 28], which exhibit some chaperone-like properties [2]. Thus, cellular or virus-coded molecular

chaperone proteins may be important during virus infection of plants. However, the characterization of plant chaperone proteins is incomplete and targeted manipulation of chaperonin genes in healthy or virus-infected plants is currently impractical. In this study, we investigated possible role(s) for molecular chaperones in the folding and assembly of TMV CP using an in vivo *E. coli*-based pseudovirus assembly system in which the functions and genetic determinants of chaperone proteins are well-characterised and amenable to manipulation.

Materials and methods

Bacterial strains

E. coli strain DH5α [29] was used for all plasmid constructions. *E. coli* strain W3110 [29] was used as a control. Several chaperone-deficient strains (kindly gifted by Dr. Anthony Gatenby, E.I. DuPont de Nemours & Co., Wilmington, Delaware) were also used to express TMV CP [14] (Table 1 and references therein).

Plasmid constructs

Standard DNA manipulations were performed as described [29]. Two compatible plasmids were co-transformed into each *E. coli* host. The first plasmid, pTrcAL, used pTrc99A (Pharmacia-LKB Biotechnology) as a parental vector to express an N-terminal Met-Ser → Met-Ala derivative of the U1 TMV CP gene. The *Nco* I-*Pst* I fragment of pAla2 [20] was inserted into the same sites in pTrc99A downstream of the *trc* promoter, to create pTrcAL (Fig. 1). pTrc99A is a ColE1 replicon which is compatible with the p15A replicons described below. pTrc99A also encodes the *lac* repressor (*lac* I), making this vector suitable for expression of TMV CP in the various chaperone-deficient *E. coli* strains used (Table 1).

Each member of a second series of plasmids (pLys105, pLys104, pLys106, pLys107) was derived from the p15A replicon pLys103 [20]. pLys103 was made from pLysE [32] by deletion of the T7 lysozyme gene. pLys105 contained an *Eco*RI fragment (0.8 kbp) from pJII102 [12], bearing the C-terminal half of the chloramphenicol acetyltransferase (CAT) gene with a DNA copy of the TMV OAS (440 bp), cloned into the *Eco*RI site in the CAT marker gene on pLys103. pLys104 comprised the same *Eco*RI fragment of pJII102 inserted into pGroESL [14] (also a gift from Dr. Anthony Gatenby, E.I. DuPont de Nemours & Co.) which contained the GroE operon controlled by the *lac* UV5 promoter. To create pLys106, pLys104 was incompletely digested with *Sac* II at either of two sites, one of which was within *groES*. The *Sac* II partial digest of pLys104 was treated with T4 DNA polymerase to make blunt ends and then self-ligated to create a frameshift mutation in *groES*. To create pLys107, pLys104 was digested to completion with *Kpn* I and self-ligated to remove the 3′

Table 1. The chaperone-deficient *E. coli* strains used in this study

CG712	(F⁻) W3110 *galE relA zid-1*::Tn*10* ***groES30***[a]	[11]
CG714	(F⁻) W3110 *galE relA* ***groEL140***	[10]
CG804	(F⁻) C600 ***grpE280***	[10, 11]
MF634	(F⁻) *thr-1* ***dnaJ259*** (Ts) *leuB6 tonA21 lacY1 supE44* λ- *rfbD1 thi-1*	[33]
MF746	(F⁻) *thr-1* ***dnaK756*** (Ts) *tonA21 lacY1 supE44* λ- *rfbD1*	[38]

[a] The chaperone-deficient genotype of each *E. coli* is in bold typeface

part of *groEL*. Thus pLys107 expressed only functional GroES. All plasmid maps are shown in Fig. 1.

ELISA to estimate yields of soluble or assembled TMV CP

Control and chaperone-deficient strains of *E. coli* were co-transformed with pTrcAL and either pLys105, pLys104, pLys106 or pLys107. Cells in mid-log phase at 30 °C were induced by addition of IPTG to 1 mM and incubated overnight. Protein extracts were made as described [20]. The total protein content of bacterial cell lysates was measured using the BCA protein assay kit (Pierce). Amounts of soluble TMV CP or helically assembled TMV-like particles were measured by double-antibody sandwich (DAS)-ELISA [35] with rabbit polyclonal antiserum or a mouse monoclonal antibody specific for an epitope (neotope) present only in TMV or the TMV CP helix [7] (TMV-253P, a kind gift from Dr. Marc van Regenmortel, CNRS IBMC, Strasbourg). Average values for the amount of soluble TMV CP or assembled pseudovirus particles were calculated from eight serial dilutions of each sample.

Immunoprecipitation

E. coli W3110 cells were transformed with pTrcAL or pLys106 separately, or co-transformed with pTrcAL and pLys106. Cells grown at 30 °C to mid-log phase were induced with IPTG at 1 mM and incubated overnight. Proteins were extracted as before [20] and immunoprecipitated with anti-TMV CP antibody as described [16]. The immunoprecipitated pellets were then boiled in SDS-gel loading buffer [22] and separated by SDS-PAGE in a 12.5% (w/v) polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes and immunoblotted as described [20]. The blots were probed sequentially with anti-GroEL antibody and anti-TMV CP antibody.

Results and discussion

GroEL, DnaK and GrpE promote the folding of TMV CP for assembly

Each chaperone-deficient strain of *E. coli* was co-transformed with pTrcAL and pLys105 (Fig. 1). After induction, total protein extracts, including the insoluble fraction, were prepared and electrophoresed in an SDS-polyacrylamide gel (Fig. 2A). Soluble TMV CP (Fig. 2B) or assembled TMV-like particles (Fig. 2C) were measured by DAS-ELISA as described in Materials and methods. Control *E. coli* strain W3110 expressed TMV CP from pTrcAL at approximately 1% (w/w) of total cellular protein.

In *groEL* and, to a lesser extent, in *dnaK* mutant *E. coli* strains, we observed significant reductions in the overall expression levels of TMV CP (Fig. 2A, lanes 5 and 3, respectively) as well as in the amounts of soluble (Fig. 2B) and helically assembled nucleocapsid (Fig. 2C) forms of TMV CP. Our results demonstrate that both GroEL and DnaK have significant direct and/or indirect effects on the total expression, correct folding and assembly of TMV CP. However, since GroEL is also essential for cell viability [11], it is conceivable that mutations in *groEL* (or *dnaK*) induce pleiotropic cellular defects in vivo.

In contrast, *grpE* mutant cells expressed total TMV CP to a higher level than control *E. coli* (Fig. 2A), but reduced the level of soluble TMV CP (Fig. 2B) and assembled TMV-like particles (Fig. 2C). This suggests that GrpE has a negative effect on overall CP accumulation, but positive effects on the correct folding and assembly of TMV CP in vivo.

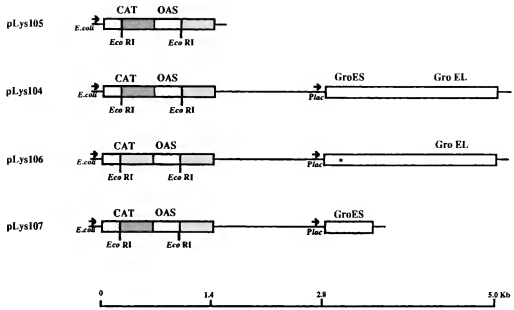

Fig. 1. Plasmids used to express TMV CP, CAT-OAS RNA or chaperone proteins in *E. coli*. Important restriction sites and genetic elements are indicated. OAS (open box) depicts the 432 bp origin-of-assembly sequence from TMV (U1 strain) RNA, CAT is chloramphenicol acetyl transferase, and bold arrows denote promoters. The asterisk indicates that the *groES* gene was destroyed by a frameshift mutation. The dotted areas in all plasmids depict a duplication of part of the CAT gene, created during cloning

In *dnaJ* mutant cells, total TMV CP expression was elevated several-fold over control *E. coli* levels (Fig. 2A), but increased less than two-fold in the soluble fraction (Fig. 2B) and in assembled TMV-like particles (Fig. 2C). These effects may arise either through increased levels of other chaperone proteins in the *dnaJ* mutant [31], or by a negative effect of DnaJ at all stages of TMV CP expression, folding and assembly in vivo.

During GroEL/GroES-mediated protein folding in vitro, the rate limiting step is the repeated binding and release of partially-folded intermediates at the GroEL/GroES complex [37] until the substrate protein is completely folded. In the presence of GroES, partially folded proteins are released slowly from GroEL by ATP hydrolysis, thus allowing them to fold properly. In the absence of GroES, proteins may be released prematurely from GroEL and form insoluble aggregates, depending on the characteristics of the substrate protein [18, 25]. Several reports have shown that GroES is not essential for the folding of some proteins such as yeast enolase [21], which are released from GroEL in the absence of GroES, in vitro.

Unexpectedly, a mutation in *groES* gave a reproducible 3- to 5-fold increase in the amounts of soluble TMV CP and assembled TMV-like particles over control *E. coli* at 30 °C (Fig. 2A), and at 25 °C or 37 °C (data not shown). As mutations in *groES* cause only a 50% increase in the levels of other heat shock proteins [31],

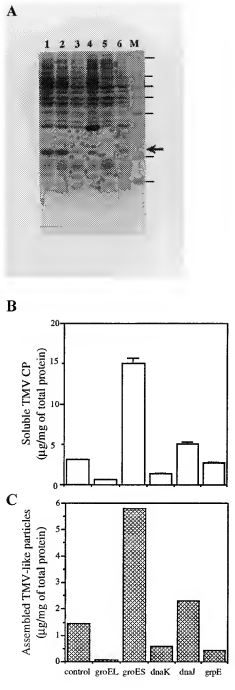

Fig. 2. Expression of total or soluble TMV CP and pseudovirus particles in chaperone-deficient *E. coli* strains. A SDS-PAGE (12.5%) of 18 h post-induction lysates (including insoluble proteins) from control (*6*) or chaperone-deficient (*1–5*) *E. coli* cells, all co-expressing CAT-OAS RNA (pLys105) and TMV CP (pTrcAL). See Table 1 for the complete genotypes of the chaperone-deficient *E. coli* strains (*1 grpE* mutant; *2 dnaJ* mutant; *3 dnaK* mutant; *4 groES* mutant; *5 groEL* mutant). Equal amounts of total protein (based on A_{600} of harvested cells) were loaded on each lane and later stained with Coomassie Blue. The position of TMV CP is indicated by the arrowhead on the right. The molecular masses of co-electrophoresed stained marker proteins (*M*) were 116, 66, 45, 36, 29, 18.4 and 14.2 kDa, respectively. Levels of soluble TMV CP (**B**) or assembled TMV CP, as pseudovirus particles (**C**), in each chaperone-deficient strain (listed below each column) were determined by DAS-ELISA of cleared cell lysates as described in Materials and methods. Amounts of total soluble protein were determined by the BCA protein assay (Pierce). Each value is the average of eight serial dilutions of each sample and error bars are shown where possible

to explain these data, GroES would also appear to have a more direct inhibitory effect on the correct folding and assembly of TMV CP (Fig. 2B, C). If TMV CP was released prematurely from GroEL without forming aggregates, then the correct folding and assembly of TMV CP could be accelerated by the absence of GroES (Fig. 2B, C).

Co-overproduction of GroEL and GroES promote TMV CP folding and assembly

Expression levels of total TMV CP, soluble TMV CP and assembled TMV-like particles were reduced most significantly by a mutation in *groEL*, and less so for *dnaK* (Fig. 2A–C). However, as these molecular chaperones are also essential for cell viability [11], we cannot conclude, unambiguously, that GroEL and DnaK have direct effects on the folding and assembly of TMV CP in vivo. To avoid pleiotropic effects due to chaperone deficiency, each chaperone which had significantly affected the correct folding and assembly of TMV CP (Fig. 2) was also overproduced in the control strain of *E. coli*.

Overproduction of GroEL and GroES together (pLys104; Fig. 1) resulted in a 2-fold increase in soluble TMV CP levels, and a 4-fold rise in assembled TMV-like particles over control cells (Fig. 3B, C), although the total amount of TMV CP expressed was similar in cells harboring pTrcAL and pLys105 (control) or pLys104 (Fig. 3A, lanes 2 and 3). Overproduction of GroEL (pLys106) or GroES (pLys107) alone not only gave similar levels of total CP (Fig. 3A, lanes 4 and 5), but also near-control (pLys105) levels of soluble TMV CP and assembled TMV-like particles (Fig. 3B,C).

GroEL/GroES are known to work synergistically during de novo protein folding [26] and in the assembly of oligomeric proteins in vitro [14]. GroEL/GroES-mediated folding is the rate limiting step [18, 26]. Co-overproduction of GroEL and GroES resulted in significantly higher yields of soluble CP and assembled TMV-like particles than overexpression of GroEL or GroES alone. Thus, together GroEL and GroES promote the correct folding of TMV CP for ribonucleocapsid assembly in *E. coli*. Moreover, because the relative levels of assembled ribonucleocapsids mirrored the levels of soluble TMV CP (Figs. 2B, C; 3B, C), we conclude that the dominant role of chaperones is to promote the correct folding and hence solubility of TMV CP, rather than assembly of quaternary structures (34-plus-mers) used in nucleocapsid formation.

Paradoxically, from the reciprocal, chaperone protein-deficient mutant experiments (Fig. 2), GroES appeared inhibitory, suggesting that overproduction of GroEL alone might greatly enhance CP expression. However, this was not so (Fig. 3).

Interaction between TMV CP and GroEL

Many proteins interact with GroEL, reflecting its fundamental role in protein folding. For example, GroEL binds to tailspike proteins in vivo [15] and a GroEL 14-mer binds to DHFR and rhodanase when they are in a "molten globule" state in vitro [25]. Such complexes can be immunoprecipitated by anti-GroEL or anti-substrate protein antibodies.

To determine whether GroEL binds TMV CP in vivo, total protein extracts from *E. coli* cells which expressed TMV CP (Fig. 4 A, B, lanes 1), or GroEL alone (Fig. 4 A, B, lanes 2), or co-expressed TMV CP (pTrcAL) and GroEL (pLys106) (Fig. 4 A, B, lanes 3) were first treated with rabbit anti-TMV CP antibody (IgG).

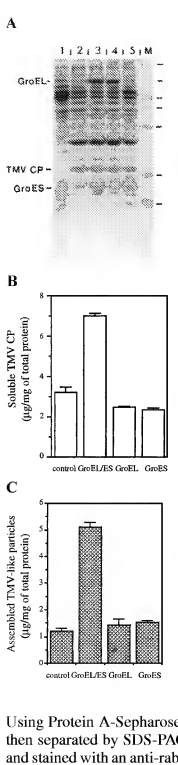

Fig. 3. Effects of (co-)overexpression of chaperone proteins in control *E. coli* W3110 cells which also expressed TMV CP and CAT-OAS RNA. **A** SDS-PAGE of an 18 h post-induction lysate (including insoluble proteins) from control *E. coli* cells expressing TMV CP (pTrcAL) and CAT-OAS RNA (pLys105; 2), or together with GroEL and GroES (pLys104; *3*), GroEL alone (pLys106; *4*), or GroES alone (pLys107; *5*). An untransformed *E. coli* W3110 cell extract is shown in *1*. The positions of TMV CP, GroEL and GroES are indicated on the left. The positions of co-electrophoresed, stained marker proteins (*6*) with molecular masses of 116, 66, 45, 36, 29, 18.4 and 14.2 kDa respectively, are shown on the right. Levels of soluble TMV CP (**B**) or assembled TMV CP as pseudovirus particles (**C**), in the presence of over-expressed chaperonin(s) (listed below each column) were determined by DAS-ELISA of cleared cell lysates as described in Materials and methods. Amounts of total soluble protein were determined by the BCA protein assay (Pierce). Each value is the average of eight serial dilutions of each sample and error bars are shown where possible

Using Protein A-Sepharose, the immunoprecipitated complexes (and IgG) were then separated by SDS-PAGE, immunoblotted with rabbit anti-GroEL antibody and stained with an anti-rabbit IgG conjugate (Fig. 4A). The blot was then probed with anti-TMV CP antibody (Fig. 4B). A band corresponding to GroEL was detected in cells which had co-expressed GroEL and TMV CP (Fig. 4 A, B, lanes 3), but not in cells expressing either GroEL or TMV CP alone (Fig. 4 A, B,

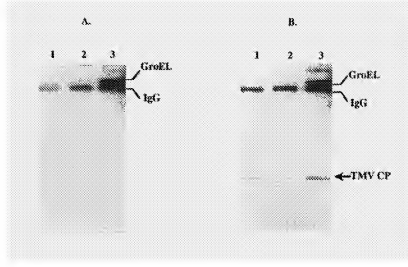

Fig. 4. Direct interaction between TMV CP and GroEL. **A** Western blot analysis, with anti-GroEL antibody, of complexes first isolated by immunoprecipitation using a rabbit polyclonal anti-TMV CP antibody from an extract of *E. coli* cells which had expressed TMV CP alone (*1*), GroEL alone (*2*), or GroEL and TMV CP (*3*). **B** The same blot was then probed with anti-TMV CP antibody. The positions of GroEL, TMV CP, and the heavy chain of IgG are indicated

lanes 1, 2). This result demonstrates that GroEL interacts sufficiently strongly with TMV CP to cause co-immunoprecipitation. No co-immunoprecipitated GroEL was detected in cells which over-expressed TMV CP alone (lanes 1), suggesting that the endogenous GroEL level in *E. coli* W3110 was low.

We conclude that TMV CP binds directly to GroEL in vivo and that GroEL/GroES together improve the folding, solubility and hence the assembly efficiency of TMV CP into ribonucleocapsids in vivo. Although extrapolating only from null-mutant experiments, the chaperonin GrpE also appears to have a positive effect on the folding and assembly of TMV CP in vivo. To our knowledge, this is the first report of bona fide chaperone proteins affecting the folding and assembly properties of a plant viral CP in vivo.

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Authors' address: Dr. T. M. A. Wilson, Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, U.K.

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